Virology 379 (2008) 30–37

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Characterization of the contribution of spliced RNAs of hepatitis B virus to DNA synthesis in transfected cultures of Huh7 and HepG2 cells

Teresa M. Abraham [a,b], Eric B. Lewellyn [a,b], Kathleen M. Haines [a,b], Daniel D. Loeb [a,*]

[a] McArdle Laboratory for Cancer Research, University of Wisconsin School of Medicine and Public Health, Madison, WI 53706, USA
[b] Program in Cellular and Molecular Biology, University of Wisconsin, Madison, WI 53706, USA

ARTICLE INFO

Article history:
Received 5 February 2008
Returned to author for revision 3 March 2008
Accepted 18 June 2008
Available online 26 July 2008

Keywords:
Spliced RNA
Pregenomic RNA
CMV-IE promoter
Minus-strand DNA synthesis
Plus-strand DNA synthesis

ABSTRACT

Hepatitis B virus synthesizes multiple spliced RNAs that can be reverse transcribed into viral DNA. We thoroughly characterized the contribution of spliced RNAs to DNA synthesis in transfected cultures of Huh7 and HepG2 cells. We found that up to 50% of DNA within intracellular capsids is derived from five spliced RNAs. Expressing HBV P protein and pgRNA from separate plasmids and the use of the CMV-IE promoter contributes to these high levels of encapsidated DNA derived from spliced RNA. A spliced RNA called Sp1 was the predominant species expressed in both cell lines. All spliced RNAs support the synthesis minus-strand DNA and duplex linear DNA. Only one of the spliced RNAs, Sp14, supported the synthesis of relaxed circular DNA because splicing removed an important *cis*-acting sequence (hM) in the other four RNAs. Additionally, we created a variant that was deficient in the synthesis of spliced RNA and supported DNA synthesis at wild-type levels. Our results reinforce and extend the idea that a significant fraction of HBV DNA synthesized under common experimental conditions is derived from spliced RNA. It is important that their presence be considered when analyzing HBV DNA replication in transfected cell cultures.

Introduction

Hepatitis B virus (HBV), a member of the *Hepadnaviridae* family, causes both acute and chronic infections of the liver. Chronically infected individuals have an increased risk of developing severe liver diseases such as cirrhosis and hepatocellular carcinoma (Beasley, 1988). Maintenance of a chronic state requires the 3.2 kb genome to be maintained in the nucleus of the hepatocyte as double-stranded, covalently closed circular (ccc) DNA (Tuttleman et al., 1986). Host RNA polymerase II transcribes all viral RNAs from cccDNA, which are classified as either unspliced or spliced. The unspliced RNAs consist of the pregenomic (pg) RNA, the precore mRNA and the subgenomic (sg) RNAs. These RNAs are transcribed from different promoters and hence have different 5′ ends but share a common 3′ end. The pgRNA, which is 3.5 kb, is the mRNA for the core (C) and the polymerase (P) proteins. The precore RNA is the mRNA for HBeAg, a secreted protein (Ou et al., 1986). The sgRNAs comprise a set of 2.4 kb, 2.1 kb and 0.9 kb RNAs. The L protein is translated from the 2.4 kb RNA, whereas the M and S proteins are translated from two different 2.1 kb RNAs. The X protein is translated from the 0.9-kb mRNA. The pgRNA is the only unspliced RNA that is encapsidated into core particles and reverse transcribed into DNA (Nassal et al., 1990; Summers and Mason, 1982).

When we performed Southern blotting on intracellular capsids from transfected cultures of Huh7 or HepG2 cells, a more complicated profile was seen when compared to duck hepatitis B virus (DHBV) (Fig. 1A). For DHBV most, if not all, encapsidated replicative intermediates are derived only from the pgRNA. For HBV, this is not the case. Several DNA species migrating faster than single-stranded (SS) DNA were seen when native or denatured HBV replicative intermediates were analyzed by Southern blotting (Fig. 1A, right panel). We undertook this study to characterize this complex profile of replicative intermediates seen during HBV replication in our cell cultures.

For HBV, in addition to pgRNA, several spliced RNA transcripts are also encapsidated and reverse transcribed into DNA. To date, at least 12 spliced RNA transcripts derived from the same promoter as pgRNA have been described (Gunther et al., 1997; Rosmorduc et al., 1995; Sommer et al., 2000; Su et al., 1989a; Suzuki et al., 1990; Suzuki et al., 1989; Terre et al., 1991; Wu et al., 1991). These spliced RNA transcripts contain the encapsidation signal, ε, and have different introns removed. The presence of the encapsidation signal in the spliced RNA transcripts permits the packaging of these RNAs into capsids when C and P proteins are present. One transcript, called Sp1 (as classified by Gunther et al., 1997), is the major spliced RNA in multiple reports. Sp1 RNA has a 1.0 kb intron from nt 2446 to 486 (inclusive). Sp1 RNA has the potential to encode core protein with the terminal cysteine residue deleted; it does not encode functional P and envelope proteins (Gunther et al., 1997; Su et al., 1989a). Sp1 RNA and DNA reverse transcribed from Sp1 RNA have been detected in

* Corresponding author. Fax: +1 608 262 2824.
E-mail address: loeb@oncology.wisc.edu (D.D. Loeb).

0042-6822/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2008.06.021

both liver tissue and sera from HBV-infected patients, and transfected cell cultures (Su et al., 1989a; Su et al.,b; Terre et al., 1991; Wu et al., 1991).

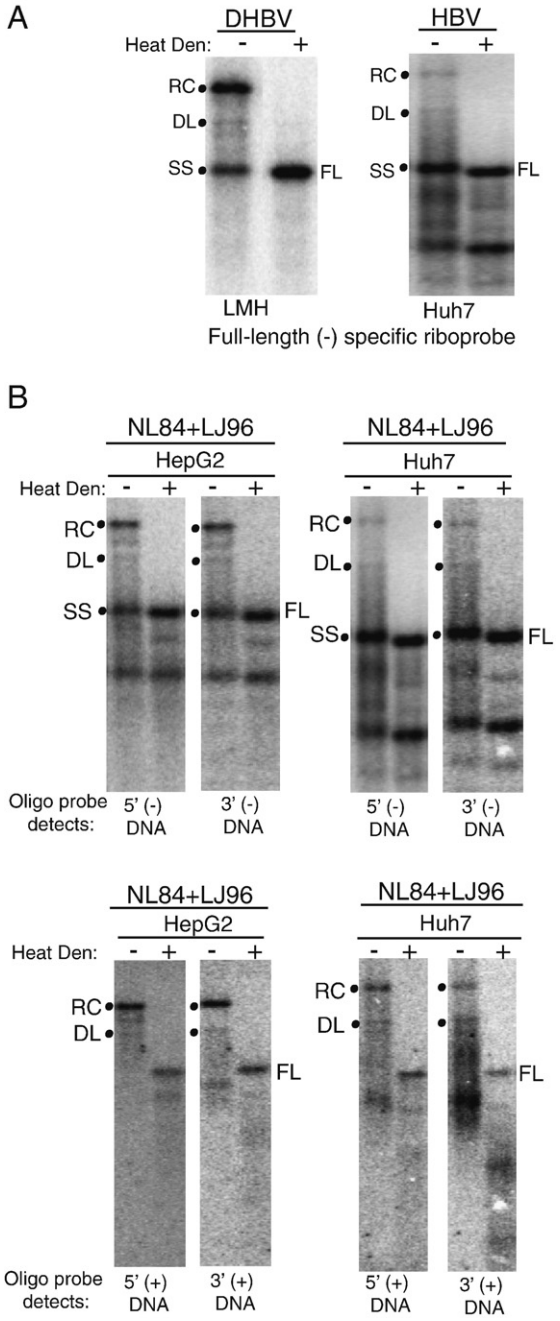


Fig. 1. The profile of HBV replicative intermediates isolated from cytoplasmic capsids is more complex than those of DHBV. (A) Southern blot of replicative intermediates isolated from cytoplasmic capsids of HBV and DHBV. Left panel represents DHBV replicative intermediates isolated from LMH cells transfected with plasmid D1.5G. Right panel represents HBV replicative intermediates isolated from Huh7 cells co-transfected with plasmids NL84 and LJ96. Samples were either untreated (native) or heat denatured before electrophoresis. Relaxed circular (RC), duplex linear (DL) and single-stranded (SS) DNA species are indicated. The blots were hybridized with a full-length, minus-strand-specific riboprobe for either DHBV or HBV. The black dots to the left of the blot indicate the location of RC, DL and SS DNA. (B) Southern blot of HBV replicative intermediates isolated from intracellular capsids from cultures of HepG2 or Huh7 cells co-transfected with plasmids NL84 and LJ96. Top panels have been hybridized with a minus-strand-specific oligonucleotide complementary to the 5′ end (ayw1661^{+}) or 3′ end (ayw 1995^{+}) of minus-strand DNA. Bottom panels have been hybridized with a plus-strand-specific oligonucleotide complementary to the 5′ end (ayw1948^{-}) or 3′ end (ayw1530^{-}) of plus-strand DNA. The black dots to the left of the blot indicate the location of RC, DL and SS DNA.

A common experimental method to study nucleic acid replication of HBV is to transfect cultures of Huh7 or HepG2 cells with separate plasmids that express the replication proteins (C and P) or the replication template (pgRNA). Under these conditions, both pgRNA and spliced RNAs will be expressed, encapsidated and reverse transcribed into DNA. If spliced RNA differs from pgRNA in its ability to be reverse transcribed into minus-strand and plus-strand DNA, then our ability to elucidate the contributions of *cis*-acting sequences and *trans*-acting factors to the replication of pgRNA will be impacted. To this end, we identified and quantitated the level of spliced RNA and DNA derived from these RNAs made in our cell cultures. Also, we made recombinant plasmids that expressed each of the spliced RNAs individually when transfected into cell cultures and characterized their ability to support the synthesis of minus-strand and plus-strand DNA. Finally, we created a variant that made undetectable levels of spliced RNA. This variant synthesized normal levels of minus-strand and plus-strand DNA.

Results

Profile of HBV replicative intermediates isolated from cytoplasmic capsids is more complex than those of DHBV

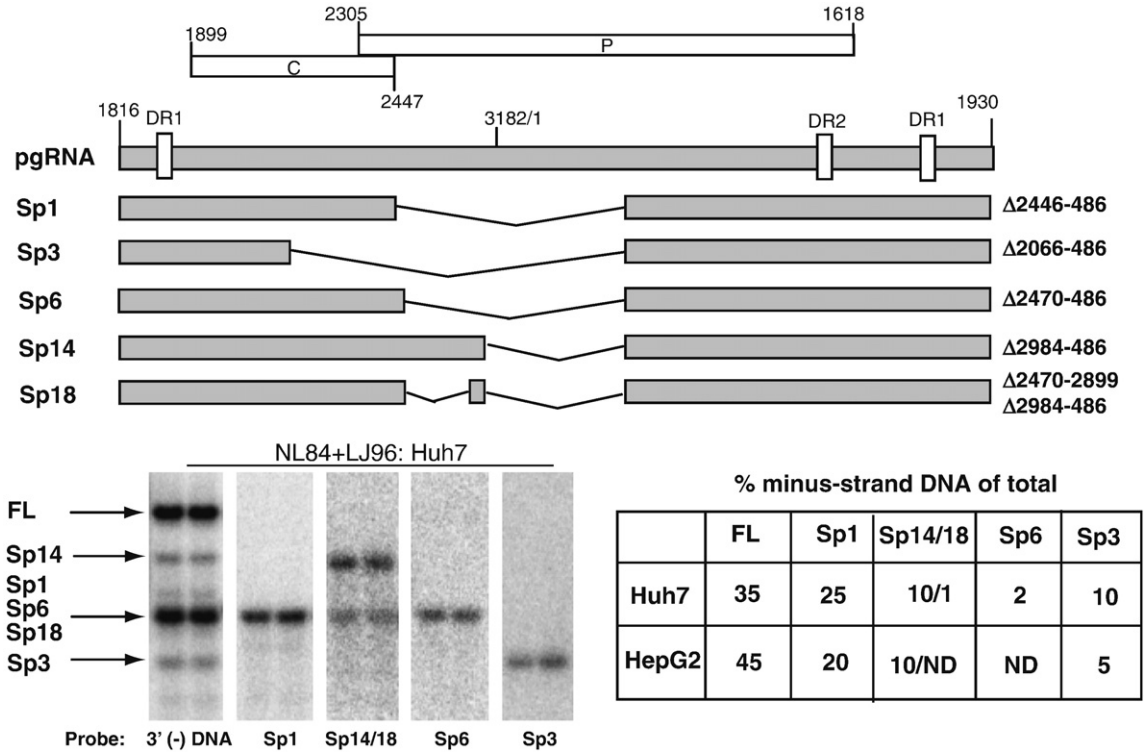
Southern blot analysis of native replicative intermediates of DHBV isolated from transfected LMH cells shows a characteristic pattern that is comprised of three major forms, RC, DL and SS DNA. Each of these three intermediates contains a full-length (FL) minus-strand DNA. Upon heat denaturation of replicative intermediates, Southern blot analysis showed a single band that corresponds to FL minus-strand DNA (Fig. 1A, left panel). In contrast, native and denatured DNA isolated from intracellular HBV capsids from cultures of transfected Huh7 cells showed a complex pattern (Fig. 1A, right panel), with several unidentified DNA species in addition to FL DNA. To understand and characterize these unidentified bands, end-labeled oligonucleotide *ayw*1661^{+} and *ayw*1995^{+} were used as hybridization probes to detect the sequences at the 5′ and 3′ ends, respectively, of minus-strand DNA (Fig. 1B, top panels). This result indicated that the DNA species had the 5′ and 3′ ends found in FL minus-strand DNA, suggesting that they were neither a result of incomplete elongation of minus-strand DNA nor the result of use of an internal acceptor site during the template switch during minus-strand DNA synthesis.

Southern blot analysis with oligonucleotides *ayw*1948^{-} and *ayw*1530^{-}, which hybridize close to the 5′ and 3′ ends of plus-strand DNA, respectively, detected fewer discrete bands (Fig. 1B, bottom panels). The majority of DNA detected corresponds to the full-length plus-strand of RC and DL DNA. In Huh7 and HepG2 cells, plus-strand DNA that migrated faster than FL plus-strand DNA was detected with both the 5′ and 3′ end probes (Fig. 1B, bottom right panels).

Identification of different DNA species from intracellular capsids

To identify the species that migrated faster than FL minus-strand DNA, we isolated intracellular replicative intermediates from Huh7 and HepG2 cells transfected with plasmids NL84 and LJ96, and performed PCR using oligonucleotides *ayw*1816^{+} and *ayw*1821^{-}. These PCR products were cloned into the HindIII (nt 1910) and BmgBI (nt 1600) restriction sites of plasmid NL84 (see Materials and methods and Table 2 for description of plasmids).

We recovered five different plasmids that were missing different portions of the HBV genome (Fig. 2). The deleted sequences corresponded precisely to introns previously seen in spliced RNAs or the DNAs derived from spliced RNA (Gunther et al., 1997; Su et al., 1989a; Suzuki et al., 1990). Sp1, 3, 6 and 14 all have a single intron removed. Sp18 removes two previously-described introns in a combination not reported to date. All five species use the splice acceptor site at nt 485. We did not recover any recombinant plasmids containing the



% minus-strand DNA of total

	FL	Sp1	Sp14/18	Sp6	Sp3
Huh7	35	25	10/1	2	10
HepG2	45	20	10/ND	ND	5

Fig. 2. Five species of DNA derived from spliced RNA were detected in replicative intermediates from intracellular capsids. (A) Schematic representation of pgRNA. The locations of the C and P genes are indicated. The white vertical boxes represent the direct repeat (DR) sequences, DR1: nts 1822–1832 and DR2: nts 1588–1598. The 5′ end of pgRNA is nt 1816. The poly(A) tail begins at approximately nt 1930. The deletions generated by splicing are indicated by thin black lines. The nucleotide coordinates of the deleted sequences are inclusive. (B) Southern blot of denatured HBV replicative intermediates isolated from intracellular capsids from Huh7 cells transfected with plasmids NL84 and LJ96. The membrane was hybridized sequentially with minus-strand-specific oligonucleotides that anneal to the 3′ end of all minus-strand DNAs and to the splice junctions of the various spliced RNAs. To detect all minus-strand DNA, oligonucleotides ayw1995^{+}; Sp1, 2445/487^{+}; Sp3, 2066/487^{+}; Sp6, 2469/487^{+}; Sp14 and 18, 2983/487^{+}, were used. Linearized plasmids were used as markers for size, specificity, efficiency of probing and as mass standards. Each spliced form was expressed as a percent of total minus-strand DNA for both Huh7 and HepG2 cells. Total minus-strand DNA is the measurement of all species between, and including, full-length (FL) and Sp3 minus-strand DNA. The positions of FL minus-strand DNA, Sp1, 3, 6, 14 and 18 minus-strand DNA are indicated. Percent minus-strand DNA for Sp6 and Sp18 in HepG2 cells were not determined.

previously-described splice acceptor site at nt 280 (see Gunther et al., 1997 for a comprehensive description of spliced donor and acceptor sequences used by HBV).

To measure the levels of each species of DNA derived from spliced RNA we used oligonucleotides that annealed to the individual splice junctions as hybridization probes. Fig. 2 shows a representative Southern blot. Oligonucleotide *ayw*1995^{+} detects all minus-strand DNA that have elongated to nt 1995 (Fig. 2). Oligonucleotides Sp1, 3, 6, and 14/18 will anneal to each of the respective splice junctions (see Table 1 for nucleotide sequence). Plasmids NL84, Sp1, 3, 6, 14 and 18 were digested with restriction enzyme Psi I, which results in double-stranded linear DNA of 3182 bp, 1959 bp, 1579 bp, 1983 bp, 2497 bp and 2067 bp, respectively. These linearized plasmids were used as markers for size, specificity of hybridization, and to normalize for efficiency of hybridization. Using the linearized plasmids as mass standards, we determined the proportion of each DNA derived from spliced RNA compared to total minus-strand DNA.

From this analysis, we found that Sp1 was the predominant DNA derived from spliced RNA within intracellular capsids in our cell cultures. Sp1 DNA represented 25% and 20% of total minus-strand DNA in our cultures of Huh7 and HepG2 cells, respectively. FL minus-strand DNA represented 35% and 45% of total minus-strand DNA in Huh7 and HepG2 cell cultures, respectively. Sp14 constitutes approximately 10% of total minus-strand DNA in both cell lines. Sp3 represents 5% and 10% of total minus-strand DNA in HepG2 and Huh7 cells, respectively. Using this approach, we accounted for approximately 80% of minus-strand DNA within capsids.

Table 1
Oligonucleotides used as hybridization probes to detect minus-strand or plus-strand DNA in Southern and northern blotting analyses

Name	Detects	Sequence
ayw1661^{+}[a]	5′ end (−)	CTCTTGGACTCTCAGCAATGTCAAC
ayw1995^{+}[a]	3′ end (−)	ACCGCCTCAGCTCTGTATCG
Sp1: 2445/487^{+}[b]	Sp1 (−)	GGGAATCTCAAT/GATCCTCAACAA
Sp3: 2065/487^{+}[b]	Sp3 (−)	ACTGCACTCAG/GATCCTCAAC
Sp6: 2469/487^{+}[b]	Sp6 (−)	TGGACTCATAAG/GATCCTCAAC
Sp14/18: 2983/487^{+}[b]	Sp14 and Sp18 (−)	GCCAACAAG/GATCCTCAAC
ayw1948^{-}[c]	5′ end (+)	GAGAGTAACTCCACAGTAGCTCC
ayw1530^{-}[d]	3′ end (+)	GAGGTGCGCCCCGTGGTCGGTCG
ayw2007^{-}[c]	5′ end (+)	GAGCTGAGGCGGTATCTAGAA

[a] Detects 5′ or 3′ end of full-length and all splice-derived minus-strand DNA.
[b] The coordinates indicate the nucleotide position of each splice junction. Backslash (/) indicates the splice junction for probes used to detect specific splice-derived (−) DNA.
[c] Detects pgRNA, spliced RNA, and the 5′ end of full-length and all splice-derived plus-strand DNA.
[d] Detects the 3′ end of plus-strand DNA derived from pgRNA and spliced RNA.

Expressing pgRNA and the replication proteins from separate plasmids lead to an increase in DNA derived from spliced RNA

To determine whether the levels of DNA derived from spliced RNA were influenced by the design of our experiments, we examined two parameters. First, we examined our strategy of providing the replication proteins and pgRNA from separate plasmids (NL84 + LJ96; see Table 2 for description) to engender replication. Normally, P protein preferentially encapsidates the pgRNA from which it is translated (Bartenschlager et al., 1990). In our system pgRNA transcribed from plasmid NL84 is not the mRNA for the replication proteins, P and C, due to premature stop codons in those genes (Liu et al., 2004). These

Table 2
HBV expressions plasmids, pgRNA promoter, and genotype

Plasmid name	pgRNA promoter	Genotype[a]
NL84	CMV IE	$\varepsilon^{+}C^{-}P^{-}S^{-}X^{-}$
EL49	CMV IE	$\varepsilon^{+}C^{-}P^{-}S^{-}X^{-}SpI^{-}$
LJ96	CMV IE	$\varepsilon^{-}C^{+}P^{+}S^{-}X^{+}$
LJ140	HBV	$\varepsilon^{+}C^{+}P^{+}S^{-}X^{+}$
LJ144	CMV IE	$\varepsilon^{+}C^{+}P^{+}S^{-}X^{+}$

[a] Status of genetic elements within each plasmid. + indicates functional, − indicates inactivated by mutation. ε — RNA encapsidation signal, C — C gene, P — P gene, S — envelope gene, X — X gene, and SpI^{-}indicates mutation to inactivate splice acceptor site at nt 487.

proteins are translated from an RNA transcribed from plasmid LJ96, which is not encapsidated, due to deletion of ε (Liu et al., 2004). To determine whether the high levels of DNA derived from spliced RNA were due to a loss of preferential encapsidation of the pgRNA, we compared replication in cells transfected with NL84 and LJ96 (in *trans*) to replication in cells where P and C proteins were translated from the same pgRNA (in *cis*: LJ144).

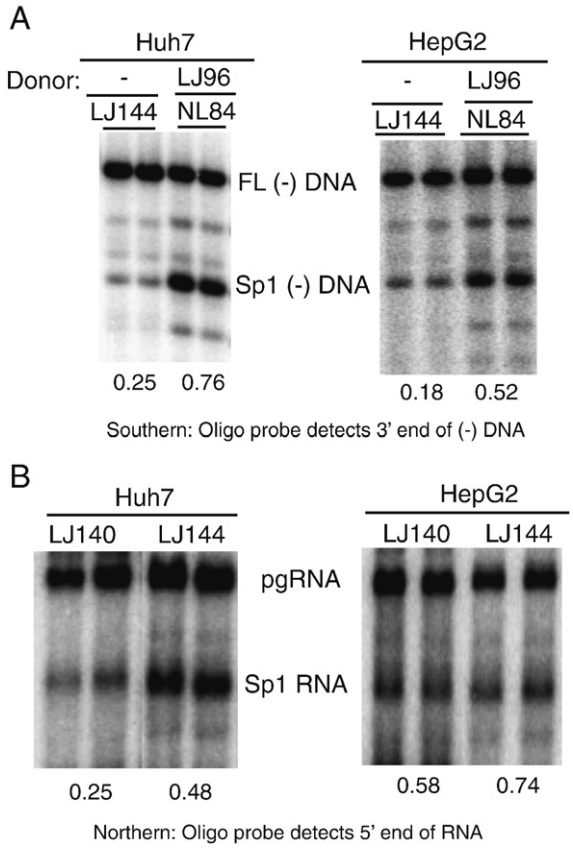


Fig. 3. The method to engender replication influences the levels of spliced RNA and subsequent DNA synthesis. (A) Southern blot of replicative intermediates isolated from intracellular capsids from Huh7 and HepG2 cells. Cells were transfected with either plasmid LJ144 (*cis*) or plasmids NL84 and LJ96 (*trans*). The CMV-IE promoter drives RNA transcription from plasmids LJ144, NL84 and LJ96. The two lanes represent duplicate samples. An oligonucleotide ($ayw1995^{+}$) complementary to the 3′ end of minus-strand DNA was used as a hybridization probe. The positions of FL minus-strand DNA and Sp1 minus-strand DNA are indicated. The ratio of Sp1 to FL minus-strand DNA is indicated below the Southern blots. FL, full-length. (B) Northern blot of total cellular, poly(A)-selected RNA isolated from Huh7 and HepG2 cells. Cells were transfected with either LJ144 or LJ140 plasmids. The CMV-IE promoter drives transcription from plasmid LJ144. Endogenous HBV promoter drives transcription from plasmid LJ140. One-third (LJ140) or 1/10 (LJ144) of the RNA isolated from a 60mm plate was analyzed by northern blotting. An oligonucleotide ($ayw2007^{-}$) complementary to the 5′ end of pgRNA and spliced RNAs was used as hybridization probe. The pgRNA and Sp1 RNA are indicated. The ratio of Sp1 RNA to pgRNA is indicated below the northern blots.

DNA was isolated from intracellular capsids from HepG2 and Huh7 cells and Southern blotting was performed on denatured replicative intermediates. The membrane was hybridized with oligonucleotide $ayw1995^{+}$, which detects minus-strand DNA derived from both spliced RNA and pgRNA (Fig. 3A). We compared the ratio of Sp1 DNA to FL DNA. In both HepG2 and Huh7 cells, the proportion of DNA derived from spliced RNA increased 2- to 3-fold when the replication proteins were provided in *trans*. This increase in the ratio of Sp1 to FL minus-strand DNA cannot be attributed to an increase in the level of Sp1 RNA. Total poly(A)-selected RNA from cell cultures transfected with plasmids LJ144 or NL84 showed that the ratios of Sp1 RNA to pgRNA were similar (data not shown).

CMV-IE promoter increases the proportion of spliced RNA

Next, we examined whether the promoter used to drive transcription of the pgRNA and spliced RNA influenced their proportions. The CMV-IE promoter is widely used to express pgRNA because it leads to higher levels of pgRNA than the endogenous HBV promoter in cell cultures. A recent study observed an increase in levels of splicing of retroviral RNA when transcription was under the control of the CMV-IE promoter instead of the retroviral long terminal repeat (LTR) (Bohne et al., 2007). To test whether the use of the CMV-IE promoter enhances accumulation of spliced RNA, we compared levels of Sp1 RNA to pgRNA from cells transfected with plasmids LJ140 (HBV promoter) or LJ144 (CMV promoter). Total poly(A)-selected RNA was isolated from Huh7 and HepG2 cells and Northern blotting was performed. The membrane was hybridized with oligonucleotide $ayw2007^{-}$, which detects a sequence close to the 5′ end of pgRNA and spliced RNA. This probe does not detect sgRNA. In cultures of Huh7 cells the ratio of Sp1 RNA to pgRNA increased approximately two-fold (Fig. 3B). In HepG2 cell cultures the increase in splicing was modest, but statistically significant.

Taken together, our results indicate that the relative level of DNA derived from spliced RNA is dependent on at least two factors: the promoter used to drive transcription of pgRNA and whether P protein is provided in *cis* or in *trans*. Although Huh7 and HepG2 cells display similar trends, HepG2 cells appear to produce a higher proportion of spliced RNA to pgRNA than Huh7 cells (Fig. 3B). Conversely, DNA derived from spliced RNA is more abundant in Huh7 cells as compared to HepG2 cells (Fig. 3A).

All spliced RNAs give rise to DL DNA; only Sp14 RNA can be reverse transcribed into RC DNA

The presence of DNA derived from spliced RNAs can complicate the study of DNA synthesis. For example, if the spliced RNAs were not efficiently converted into RC DNA, then their presence could complicate studies of RC DNA synthesis. To understand whether spliced RNAs were converted into minus-strand and plus-strand DNA as

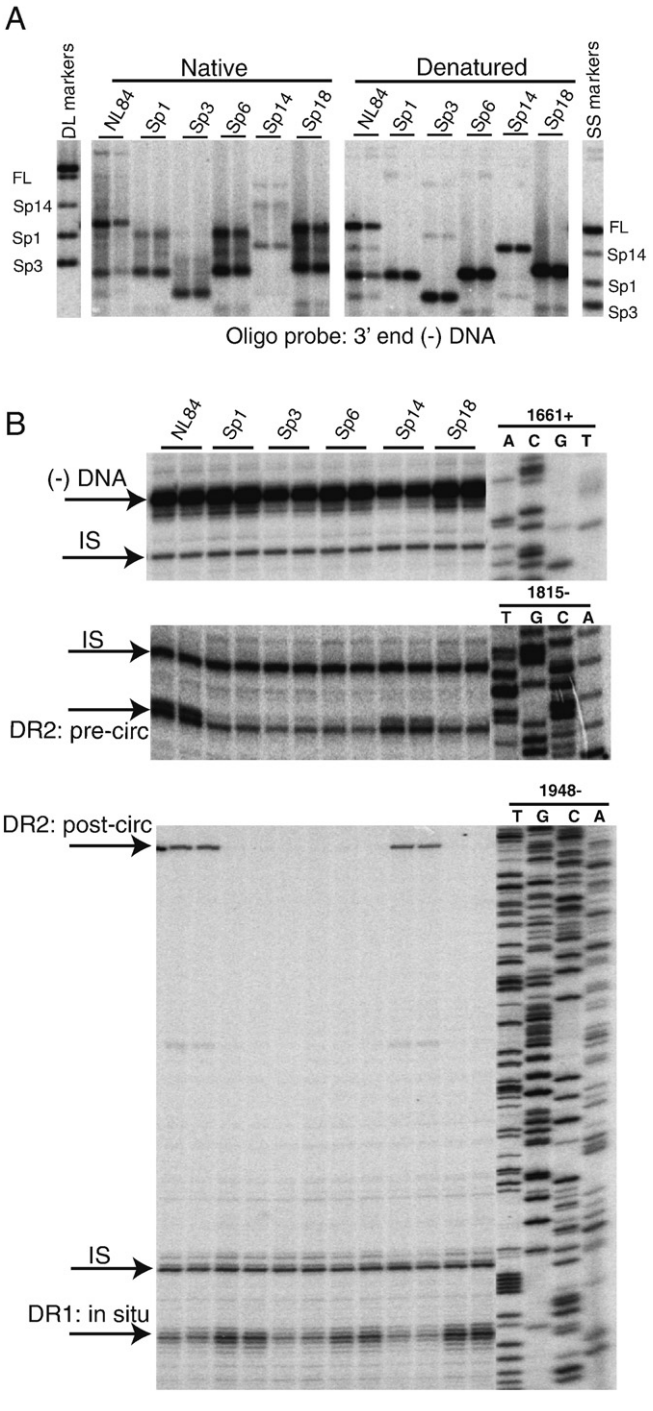
Table 3
Efficiency of minus-strand and plus-strand DNA synthesis of HBV DNA in Huh7 cells relative to NL84 (WT = 100)

	(−) DNA synthesis[a]	Priming from DR2[b]	Circularization[b]	Priming from DR1[b]	Total priming[b]
NL84 (WT)	100	100	100	100	100
EL49 (Sp^{-})	102±1	127±8	116±3	80±3	106±5
Sp1	98±4	25±4	ND[c]	135±34	75±12
Sp3	88±5	25±10	ND	116±26	68±15
Sp6	104±8	23±5	ND	138±42	76±17
Sp14	116±4	117±19	117±28	127±7	123±11
Sp18	110±12	25±6	ND	152±48	83±19

[a] Measured by primer extension with two oligonucleotides as described previously (Abraham and Loeb, 2006).
[b] Measured by primer extension with one oligonucleotide as described previously (Lewellyn and Loeb, 2007; Haines and Loeb, 2007).
[c] ND—none detected.

efficiently as pgRNA, we used expression plasmids for the specific spliced forms and analyzed their ability to support the synthesis of DNA in cell culture. To analyze the ability of the spliced RNAs to be converted into minus-strand DNA, encapsidated replicative intermediates were isolated and primer extension was performed to measure the level of minus-strand DNA as a function of encapsidated pgRNA (Table 3) (Abraham and Loeb, 2006). To measure the level of priming of plus-strand DNA from DR2 and DR1, primer extension was performed (Fig. 4B), as described (Lewellyn and Loeb, 2007). Table 3 summarizes the ability of each of the spliced RNAs to support the synthesis of minus- and plus-strand DNA.

We learned that once a spliced RNA is packaged into a capsid it is reverse transcribed into minus-strand DNA as efficiently as the WT reference (Table 3). Based on Southern blotting, we determined that although all spliced forms were converted into DL DNA, only Sp14 RNA was converted into RC DNA (Fig. 4A). Synthesis of the plus-strand of RC DNA requires two template switches, primer translocation and circularization (Lien et al., 1986). Both primer translocation and circularization are a result of priming from DR2. Using primer extension to analyze Sp14, we learned that the levels of priming from DR2 (DR2: pre-circ) and circularization (DR2: post-circ) were at the same level as the WT reference (Fig. 4B). In contrast, priming from DR2 for Sp1, 3, 6 and 18 was at 25% the level of the WT reference (Table 3 and Fig. 4B, DR2: pre-circ), and no circularization was detected (Fig. 4B, DR2: post-circ). All spliced forms displayed higher than WT levels of priming from DR1 (Fig. 4B, DR1: in situ).



Mutation of the splice acceptor site abrogates splicing

To simplify our analyses, we sought to remove these spliced RNAs from our cell cultures. We changed four nucleotides within the splice acceptor site at nt 485 (plasmid EL49) (Fig. 5A). Northern blotting of total poly(A)-selected RNA showed that EL49 did not express detectable levels of Sp1 RNA. Southern blotting of replicative intermediates from intracellular capsids showed that DNA derived from Sp1, Sp3, Sp6, Sp14, and Sp18 RNA was not detected (Fig. 5B and data not shown) although there were still several faster-migrating DNA species present at low levels. The identity of these DNA forms is presently unknown. The mutation in EL49 did not affect minus- and plus-strand DNA synthesis (Table 3).

Discussion

This study reveals that the gamut of HBV replicative intermediates is not as simple as that for DHBV, where RC, DL and SS DNA intermediates are derived from a single template, pgRNA (Fig. 1A). In our cell cultures, in addition to the canonical RC, DL, and SS DNA, HBV synthesizes multiple additional species of DNA (Fig. 1A). We demonstrate that most of these DNA species arise from reverse transcription of spliced RNA. These spliced RNA transcripts are encapsidated and reverse transcribed into minus-strand DNA. All spliced forms are converted into DL DNA, but only Sp14 RNA is converted into RC DNA. The presence of these spliced forms of DNA can complicate the study of HBV DNA synthesis if not recognized and taken into account. In the reported literature it is not uncommon for us to see misidentification the DNA forms in Southern blot analysis of intracellular capsid DNA. Usually this entails identifying the major spliced species as SS DNA and authentic SS DNA as DL DNA. An unrecognized misidentification like this would indicate higher levels of DL than RC DNA, a situation that is implausible, if not impossible, for WT HBV.

Fig. 4. All spliced RNAs support the synthesis of DL DNA, only Sp14 RNA engenders the synthesis of RC DNA. (A) Southern blot of replicative intermediates from intracellular capsids isolated from Huh7 cells transfected with either pgRNA expression plasmid (NL84) or each of the spliced RNA expression plasmids and the donor plasmid (LJ96). Panels containing either native or heat denatured samples are indicated. Markers for duplex linear and single-stranded DNA are shown on the left and right, respectively. An oligonucleotide (ayw 1995^{+}) complementary to the 3′ end of minus-strand DNA was used as hybridization probes. (B) Primer extension analysis with oligonucleotides 1661^{+} (top), 1815^{-} (middle) and 1948^{-} (bottom). Oligo 1661^{+} was used to measure the level of minus-strand DNA and anneals 164 nt from the its 5′ end. Oligo 1815^{-} was used to measure the level of plus-strand priming from DR2 and anneals 217 nt from DR2 at a position prior to the circularization point. Oligo 1948^{-} was used to measure the level of plus-strand priming from DR1 and the level of circularized plus-strands that initiated from DR2. It anneals 116 nt from DR1 and 126 nt from the circularization point. Sequencing ladder is on the right. Bands representing internal standard (IS), minus-strand (−) DNA, priming from DR2:pre-circ, priming from DR2:post-circ and priming from DR1:in situ are indicated on the left. The signal for each HBV-derived band was normalized to the signal for its internal standard. The level of priming from DR2 was calculated as the level of DR2:pre-circ divided by (−) DNA. Circularization was calculated as the level of DR2:post-circ divided by DR2:pre-circ. Priming from DR1 was calculated as the level of DR1:in situ divided by (−) DNA. Total priming was calculated as the sum of priming from DR2: pre-circ plus priming from DR1. All values were expressed as a percentage of a WT reference (NL84).

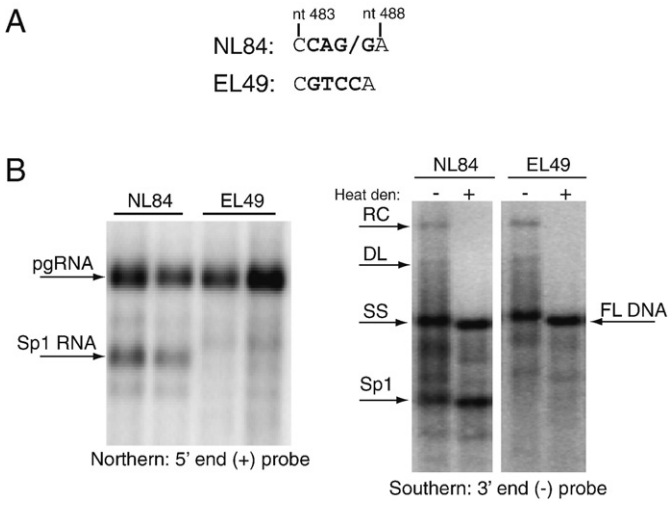


Fig. 5. Mutations in common splice acceptor site abrogates splicing. (A) Canonical sequence for 3′ splice site is indicated in bold (CAGG) on plasmid NL84. The four-nt substitution within the splice acceptor site in plasmid EL49 is indicated. (B) Left panel is a northern blot of total cellular poly(A)-selected RNA from Huh7 cells transfected with plasmids NL84 (Sp^+) or EL49 (Sp^-). Sp^+ and Sp^- indicate whether the splice acceptor site is unaltered or mutated. The position of pgRNA and Sp1 RNA are indicated. The membrane was hybridized with a plus-strand-specific oligonucleotide ($ayw2007^-$) complementary to the 5′ ends of pgRNA and all spliced RNAs. This probe does not detect sgRNAs. The right panel is a Southern blot of DNA from Huh7 cells co-transfected with plasmids NL84 or EL49 and donor plasmid, LJ96. The samples are either native or heat denatured. The position of RC, DL and SS are indicated for native samples. The position of FL minus-strand DNA and Sp1 minus-strand DNA is indicated for heat denatured samples. The Southern blot was hybridized with an oligonucleotide ($ayw1995^+$) complementary to the 3′ end of minus-strand DNA. FL, full-length; RC, relaxed circular; DL, duplex linear; SS, single-stranded.

We measured the level of DNA derived from spliced RNA within intracellular capsids using oligonucleotides specific for each splice junction as hybridization probes in Southern blotting. We characterized five spliced RNA transcripts, Sp1, 3, 6, 14 and 18, that are synthesized, encapsidated and reverse transcribed into DNA in our cell cultures. Our study shows that for the *ayw* subtype (genotype D), Sp1 is the predominant DNA derived from spliced RNA detected within intracellular capsids in both Huh7 and HepG2 cell cultures. Sommer et al. (Sommer et al. (2000) also found that Sp1 was the major spliced RNA transcribed in HepG2 and Huh7 cell cultures transfected with HBV genotypes C, D and E.

We have shown that the high levels of DNA derived from spliced RNA are in part due to the methods used to engender replication. When the replication proteins were translated from an mRNA (LJ96) other than the pgRNA, the ratio of DNA derived from spliced RNA to DNA derived from pgRNA increased 2.4- and 3.5-fold in HepG2 and Huh7 cells, respectively, as compared to when the replication proteins were translated from the pgRNA (LJ144) (Fig. 3A). This increase in the proportion of DNA derived from spliced RNA could be the result of enhanced transcription/splicing, increases in encapsidation of spliced RNA or the efficiency of minus-strand DNA synthesis. Analysis of total poly(A)-selected RNA isolated from cell cultures transfected with plasmids LJ144 or NL84 showed that the ratio of Sp1 RNA to pgRNA was similar (data not shown). This result suggests that differential transcription/splicing from plasmids LJ144 and NL84 does not contribute to the increase in DNA derived from spliced RNA. Also, each spliced RNA supports minus-strand DNA synthesis to the same level as pgRNA (Table 3). Therefore, the increase in levels of DNA derived from spliced RNA likely reflects a relative increase in encapsidation of spliced RNA when replication proteins are provided in *trans* (NL84 + LJ96).

We found that the promoter used to engender transcription of pgRNA also influences the levels of spliced RNA in cell culture. There is precedence for this finding. Several reports indicate that the choice of promoter affects levels of splicing by decreasing the rate of elongation of the RNA polymerase II or by recruiting different splicing factors to the RNA transcription complex (Auboeuf et al., 2002; Bohne et al., 2007; Cramer et al., 1997; de la Mata et al., 2003). In Huh7 cells, a two-fold increase in splicing was seen when the CMV-IE promoter was used to engender transcription when compared to the endogenous HBV promoter (Fig. 3B). In HepG2 cells, this increase was 1.3-fold. Also, the relative levels of splicing of HBV RNA differed in Huh7 and HepG2 cells. In HepG2 cells, there is a two-fold increase in spliced RNAs as compared to Huh7 cells, independent of promoter (Fig. 3B). This difference in the ratio of spliced RNA to pgRNA is likely a reflection of differences in the synthesis or degradation of HBV RNA between the two cell lines.

When we analyzed cells transfected with plasmids that individually expressed each spliced RNA transcript, we learned that all supported the synthesis of minus-strand DNA as well as the wild-type reference (Table 3). In addition, all spliced RNA transcripts supported the synthesis of DL DNA (Fig. 4A). Only Sp14 RNA engendered the synthesis of RC DNA (Fig. 4A). Primer extension analysis showed that Sp14 undergoes both primer translocation and circularization to the same extent as the WT reference (Fig. 4B and Table 3). Sp1, 3, 6 and 18 undergo primer translocation at about 25% the level of the WT reference with no detectable circularization. This result is expected because the introns spliced from Sp1, 3, 6 and 18 RNA remove a required *cis*-acting sequence, hM, for the synthesis of RC DNA (Lewellyn and Loeb, 2007). Although all spliced RNAs support slightly higher levels of plus-strand priming from DR1 (approximately 1.2- to 1.5-fold higher than the WT reference), the total level of priming of plus-strand DNA was lower for all spliced forms, with the exception of Sp14 (Table 3).

Interestingly, Sommer et al. detected lower levels of Sp1 DNA in extracellular virions when compared to full-length DNA (Sommer et al., 2000). Because only capsids containing mature DNA are enveloped (Gerelsaikhan et al., 1996), they postulated that the apparent decrease in secretion of virions containing Sp1 DNA could be the result of lower levels of plus-strand DNA (Sommer et al., 2000). Our results indicate that synthesis of plus-strand DNA is inefficient for Sp1. We predict that capsids containing Sp14 DNA will be enveloped and secreted from cells as efficiently as ones containing full-length DNA, unless the size of the genome has an impact on envelopment.

We were able to abrogate levels of splicing by altering the common splice acceptor site (Fig. 5A). The results in Fig. 5B shows that both spliced RNA and DNA derived from spliced RNAs were no longer detected and synthesis of DNA from the full-length template was normal. This variant will be a useful tool to study the contribution of *cis*-acting sequences and *trans*-acting factors to the synthesis of plus-strand DNA synthesized from full-length minus-strand DNA.

Our work has shown that DNA derived from spliced RNA can comprise up to one half of the replicative intermediates isolated from intracellular capsids in both cell lines. These high levels were influenced by the methods we used to engender DNA synthesis. Although we detected five spliced RNAs in our cell cultures, other spliced forms are likely to be present depending on HBV genotype (Gunther et al., 1997; Sommer et al., 2000). Thus, DNA derived from spliced RNA appears to be a consistent feature of HBV replication independent of expression plasmid, genotype or cell line used. Therefore, it is important to account for their presence when analyzing DNA replication in transfected cell cultures.

Materials and methods

Molecular clones

All molecular clones of HBV were derived from the subtype *ayw* (GenBank accession number V01460). The C of the unique EcoRI site (GAATTC) was designated nucleotide position 1. The molecular clone for DHBV was derived from the strain DHBV3. The plasmid D1.5G

contains 1.5 copies of the DHBV genome and expresses the DHBV pgRNA and all viral proteins (Habig and Loeb, 2002). Plasmids LJ144 and NL84 contain 1.1 copies of the HBV genome, and express the HBV pgRNA under the transcriptional control of CMV-IE promoter and derived from the parental clone pCH-9/3091 (Nassal, 1992). Plasmid LJ144 does not express the envelope proteins (Haines and Loeb, 2007). Plasmid NL84 is null for P, C, X and envelope protein synthesis (Liu et al., 2004). A separate plasmid, LJ96, provides the proteins P, C and X, in trans, but its RNA cannot be encapsidated (Liu et al., 2004). LJ96 was derived from pCH-9/3142 (Junker-Niepmann et al., 1990). The plasmid LJ140 contains 1.3 copies of the HBV genome. It expresses the HBV pgRNA under the transcriptional control of the endogenous HBV promoter but does not express any of the three envelope proteins due to mutation. Plasmid EL49 was constructed by oligonucleotide-directed mutagenesis and derived from plasmid NL84. EL49 has nts 484 to 487 changed from CAGG to GTCC. These changes disrupt the splice acceptor site (Mount, 1982). Sp1, 3, 6, 14 and 18 plasmids express RNAs with the authentic spliced junctions (Fig. 2). These plasmids were created by PCR amplification of viral replicative intermediates isolated from Huh7 or HepG2 cells using oligonucleotides *ayw*1816$^+$, which has a sequence complementary to the minus-strand from nt 1816 to 1858 on the genome, and *ayw*1821$^-$, which has a sequence complementary to the plus-strand from nt 1797 to 1821 on the genome. The amplified DNA fragments were cloned into the plasmid NL84. Splice junctions were identified by DNA sequencing and classified according to the nomenclature by Gunther et al. (1997).

Cell cultures and transfection

Chicken hepatoma cell line, LMH, was transfected with DHBV plasmids. Culturing and transfection of LMH cells were performed as described (Loeb and Tian, 1995). Human hepatoma cell lines, Huh7 and HepG2, were transfected with HBV plasmids. Culturing and transfection of Huh7 and HepG2 cells were performed as described (Liu et al., 2004).

Isolation of total cellular poly(A) RNA and encapsidated nucleic acid

Total cellular poly(A) RNA was isolated from Huh7 or HepG2 cells as described (Loeb et al., 2002). Viral nucleic acid from cytoplasmic capsids was isolated from LMH cells (Habig and Loeb, 2002) and Huh7 and HepG2 cells (Liu et al., 2004) as described.

Analysis of viral nucleic acid

One-third of the RNA isolated from a 60-mm plate transfected with the plasmid LJ140, 1/10 of the RNA isolated from the plate transfected with plasmid LJ144, and 1/6 of the RNA isolated from the plate transfected with plasmids NL84 or EL49 was analyzed by northern blotting as described (Loeb et al., 2002). These fractions represented similar mass of pgRNA. pgRNA and spliced RNAs were detected using an end-labeled oligonucleotide *ayw*2007– via hybridization of northern blots. This probe does not detect sgRNAs. See Table 1 for the nucleotide sequence of *ayw*2007$^-$. One-half of the DNA extracted from intracellular capsids from plates transfected with plasmid NL84 and 1/6 of the DNA isolated from intracellular capsids isolated from cultures transfected with plasmid LJ144 were analyzed by Southern blotting as described (Lewellyn and Loeb, 2007). These fractions contained similar masses of FL minus-strand DNA. Prior to electrophoresis, replicative intermediates were treated with 2 mg of RNase A at 37 °C for 30 min. The samples were either untreated or heat denatured at 95 °C for 3 min and placed on ice for 5 min. Samples were electrophoresed in a 1.25% agarose gel, and transferred to Hybond N membrane. ^{32}P-labeled oligonucleotide probes or ^{32}P-labeled full-length RNA probes were used to detect minus-strand DNA. ^{32}P-labeled oligonucleotides were used to detect plus-strand DNA. Membranes were incubated at a specific annealing temperature for each oligonucleotide for 4 hours, washed with Church wash buffer (1% SDS, 20 20 mM Na_2HPO_4 [pH 7.2], 1 mM EDTA [pH 8.0]) at room temperature, and placed into phosphorimaging cassettes. Membranes were probed sequentially for all minus-strand DNA, specific splice-derived minus-strand DNA and all plus-strand DNA. Oligonucleotide probes were removed from the membrane by incubating the membrane at 65 °C in H_2O for 30 min. Removal of oligonucleotide probes was verified by autoradiography. See Table 1 for nucleotide sequence of oligonucleotide probes.

Primer extension analysis with two oligonucleotides was used to measure the levels of minus-strand DNA normalized to encapsidation events for each spliced RNA as described (Abraham and Loeb, 2006).

Primer extension to measure the level of plus-strand priming from DR2, circularization of plus-strands initiating from DR2 and plus-strand priming from DR1 was performed as described (Lewellyn and Loeb, 2007). Briefly, 600 pg of a linearized plasmid was added to each sample of capsid DNA as an internal standard. The DNA mixture was heated to 95 °C for 5 min, treated with 1 mg of RNase A for 30 min at 37 °C, precipitated with ethanol, and resuspended in H_2O. This sample was split into three portions for primer extension analysis. To measure levels of minus-strand DNA, oligonucleotide *ayw*1661$^+$ with sequence complementary to nt 1661 to 1685 was used. Extension from oligonucleotide ayw1661$^+$results in a 165 nt DNA fragment. Oligonucleotide *ayw*1815$^-$ with sequence complementary to nt 1801 to 1815 was used to measure the level of plus-strand DNA that had primed from DR2 and extended to at least nt 1815. Extension using *ayw*1815$^-$ results in a 217 nt DNA fragment. Oligonucleotide *ayw*1948$^-$ with sequence complementary to nt 1928 to 1948 was used to measure the level of plus-strand DNA that had initiated from DR2 and extended to at least nt 1948. This 350-nt product with *ayw*1948$^-$ represents plus-strand DNA that had undergone circularization. Oligonucleotide 1948$^-$ also measured the level of plus-strand DNA primed from DR1 (132 nt DNA fragment). Sequencing ladders were generated using individual oligonucleotides and HBV plasmid DNA as template, as described (Habig and Loeb, 2002). Primer extension products were electrophoresed through a 5% denaturing polyacrylamide gel. The gels were dried, placed into phosphorimaging cassettes, and scanned using Molecular Dynamics Typhoon (model 8610).

Acknowledgments

We thank Megan Maguire for critically reviewing this manuscript. This work was supported by National Institutes of Health grant P01 CA22443 and T32 CA009135 (EBL).

References

Abraham, T.M., Loeb, D.D., 2006. Base pairing between the 5′ half of ε and a *cis*-acting sequence, ϕ, makes a contribution to the synthesis of minus-strand DNA for human hepatitis B virus. J. Virol 80 (9), 4380–4387.

Auboeuf, D., Honig, A., Berget, S.M., O'Malley, B.W., 2002. Coordinate regulation of transcription and splicing by steroid receptor coregulators. Science 298 (5592), 416–419.

Bartenschlager, R., Junker-Niepmann, M., Schaller, H., 1990. The P gene product of hepatitis B virus is required as a structural component for genomic RNA encapsidation. J. Virol 64 (11), 5324–5332.

Beasley, R.P., 1988. Hepatitis B virus: the major etiology of hepatocellular carcinoma. Cancer 61, 1942–1956.

Bohne, J., Schambach, A., Zychlinski, D., 2007. New way of regulating alternative splicing in retroviruses: the promoter makes a difference. J. Virol. 81 (7), 3652–3656.

Cramer, P., Pesce, C.G., Baralle, F.E., Kornblihtt, A.R., 1997. Functional association between promoter structure and transcript alternative splicing. Proc. Natl. Acad. Sci. U. S. A. 94 (21), 11456–11460.

de la Mata, M., Alonso, C.R., Kadener, S., Fededa, J.P., Blaustein, M., Pelisch, F., Cramer, P., Bentley, D., Kornblihtt, A.R., 2003. A slow RNA polymerase II affects alternative splicing in vivo. Mol. Cell. 12 (2), 525–532.

Gerelsaikhan, T., Tavis, J.E., Bruss, V., 1996. Hepatitis B virus nucleocapsid envelopment does not occur without genomic DNA synthesis. J. Virol. 70 (7), 4269–4274.

Gunther, S., Sommer, G., Iwanska, A., Will, H., 1997. Heterogeneity and common features of defective hepatitis B virus genomes derived from spliced pregenomic RNA. Virology 238 (2), 363–371.

Habig, J.W., Loeb, D.D., 2002. Small DNA hairpin negatively regulates in situ priming during duck hepatitis B virus reverse transcription. J. Virol 76 (3), 980–989.
Haines, K.M., Loeb, D.D., 2007. The sequence of the RNA primer and the DNA template influence the initiation of plus-strand DNA synthesis in hepatitis B virus. J. Mol. Biol. 370 (3), 471–480.
Junker-Niepmann, M., Bartenschlager, R., Schaller, H., 1990. A short *cis*-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. EMBO J 9 (10), 3389–3396.
Lewellyn, E.B., Loeb, D.D., 2007. Base pairing between *cis*-acting sequences contributes to template switching during plus-strand DNA synthesis in human hepatitis B virus. J. Virol 81 (12), 6207–6215.
Lien, J.M., Aldrich, C.E., Mason, W.S., 1986. Evidence that a capped oligoribonucleotide is the primer for duck heptitis B virus plus-strand DNA synthesis. J. Virol 57, 229–236.
Liu, N., Ji, L., Maguire, M.L., Loeb, D.D., 2004. *cis*-Acting sequences that contribute to the synthesis of relaxed-circular DNA of human hepatitis B virus. J. Virol 78 (2), 642–649.
Loeb, D., Tian, R., 1995. Transfer of the minus strand of DNA during hepadnavirus replication is not invariable but prefers a specific location. J. Virol 69 (11), 6886–6891.
Loeb, D.D., Mack, A.A., Tian, R., 2002. A secondary structure that contains the 5′ and 3′ splice sites suppresses splicing of duck hepatitis B virus pregenomic RNA. J. Virol 76 (20), 10195–10202.
Mount, S.M., 1982. A catalogue of splice junction sequences. Nucleic Acids Res. 10 (2), 459–472.
Nassal, M., 1992. The arginine-rich domain of the hepatitis B virus core protein is required for pregenome encapsidation and productive viral positive-strand DNA synthesis but not for virus assembly. J. Virol 66 (7), 4107–4116.
Nassal, M., Junker-Niepmann, M., Schaller, H., 1990. Translational inactivation of RNA function: discrimination against a subset of genomic transcripts during HBV nucleocapsid assembly. Cell 63 (6), 1357–1363.
Ou, J.H., Laub, O., Rutter, W.J., 1986. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. Proc. Natl. Acad. Sci. U. S. A. 83 (6), 1578–1582.
Rosmorduc, O., Petit, M.A., Pol, S., Capel, F., Bortolotti, F., Berthelot, P., Brechot, C., Kremsdorf, D., 1995. In vivo and in vitro expression of defective hepatitis B virus particles generated by spliced hepatitis B virus RNA. Hepatology 22 (1), 10–19.
Sommer, G., van Bommel, F., Will, H., 2000. Genotype-specific synthesis and secretion of spliced hepatitis B virus genomes in hepatoma cells. Virology 271 (2), 371–381.
Su, T.S., Lai, C.J., Huang, J.L., Lin, L.H., Yauk, Y.K., Chang, C.M., Lo, S.J., Han, S.H., 1989a. Hepatitis B virus transcript produced by RNA splicing. J. Virol. 63 (9), 4011–4018.
Su, T.S., Lui, W.Y., Lin, L.H., Han, S.H., P'Eng, F.K., 1989b. Analysis of hepatitis B virus transcripts in infected human livers. Hepatology 9 (2), 180–185.
Summers, J., Mason, W.S., 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell 29 (2), 403–415.
Suzuki, T., Kajino, K., Masui, N., Saito, I., Miyamura, T., 1990. Alternative splicing of hepatitis B virus RNAs in HepG2 cells transfected with the viral DNA. Virology 179 (2), 881–885.
Suzuki, T., Masui, N., Kajino, K., Saito, I., Miyamura, T., 1989. Detection and mapping of spliced RNA from a human hepatoma cell line transfected with the hepatitis B virus genome. Proc. Natl. Acad. Sci. U. S. A. 86 (21), 8422–8426.
Terre, S., Petit, M.A., Brechot, C., 1991. Defective hepatitis B virus particles are generated by packaging and reverse transcription of spliced viral RNAs in vivo. J. Virol. 65 (10), 5539–5543.
Tuttleman, J., Pourcel, C., Summer, J., 1986. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell 47, 451–460.
Wu, H.L., Chen, P.J., Lin, M.H., Chen, D.S., 1991. Temporal aspects of major viral transcript expression in Hep G2 cells transfected with cloned hepatitis B virus DNA: with emphasis on the X transcript. Virology 185 (2), 644–651.